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14. ABSTRACT

The purpose of this project is to develop aptamer-targeted DNA:Zn2+ complexes that are highly efficacious for prostate cancer treatment. Significant progress has been made on refining novel Zn2+-binding DNA motifs that utilize FdU nucleotides and that are highly cytotoxic towards prostate cancer cells. We demonstrated netropsin binding to the 3'-FdU DNA hairpin, clarified the mode of Zn2+ binding to this complex and demonstrated the Zn2+/DNA/netropsin ternary complex is highly cytotoxic towards prostate cancer cells. We also demonstrated DNA hairpins with stems consisting of G-FdU base pairs form complexes with Zn2+ that have increased stability at physiological pH relative to stems consisting of A-FdU base pairs. We completed SELEX and identified a new DNA aptamer to PSMA and demonstrated selective binding towards PSMA+ prostate cancer cells. We demonstrated that Zn2+-pyrithione is synergistic with FdUMP[10] for inducing cytotoxicity towards prostate cancer cells. We have purchased animals and are ready to evaluate Zn2+-pyrithione/FdUMP[10] in vivo in the coming months. All components are in place for forming the dimeric aptamer complexes and evaluating cytotoxicity in vitro and in vivo.

15. SUBJECT TERMS

Prostate cancer, DNA-metal interactions, Zn2+, structural biology

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INTRODUCTION: The project "Metallated DNA Aptamers for Prostate Cancer Treatment" provides information on a novel therapeutic approach for prostate cancer treatment in which Zn2+ chelates are delivered specifically to prostate cancer cells using DNA aptamers that bind to prostate specific membrane antigen (PSMA). Our preliminary data indicated that chelated Zn2+ (Zn2+-pyrithone) was cytotoxic towards human prostate cancer cells and that Zn2+-pyrithione enhanced the cytotoxicity of FdUMP[10], a novel polymeric fluoropyrimidine compound that is under investigation in Dr. Gmeiner's lab at WFUSM and at the NCI for the treatment of prostate cancer and other malignancies. The project involves developing new DNA aptamers that bind to PSMA, incorporating Zn2+-chelating DNA structural elements proximal to the PSMA-binding sequence, and forming aptamer-targeted Zn2+:DNA complexes. The resulting modified DNA aptamers are then used to form a dimeric aptamer complex composed of two monomeric aptamer complexes. Dimerization is accomplished by including a polydA tail in one aptamer complex and a polydT tail in a second aptamer complex, with dimerization occurring by Watson-Crick base pair formation. Cytotoxicity is engineered into the dimeric aptamer complex by inclusion of 5-fluoro-2'-deoxyuridine-5'-O-monophosphate (FdUMP) in place of dT in the dimerization motif. We recently demonstrated that stretches of consecutive FdU nucleotides create a unique major groove binding motif for Zn2+ (Ghosh et al., 2011; Ghosh et al., in preparation - appendix #1). This novel mode of Zn2+ binding will be used for creating the dimeric aptamer complexes. The resulting dimeric aptamer complexes will be evaluated for cytotoxicity towards prostate cancer cells in tissue culture and towards prostate cancer xenografts in nude mice as an animal model of human prostate cancer. The long-term goal of this research is to develop a highly specific, well-tolerated, and highly potent therapeutic approach for treatment of advanced prostate cancer.

BODY:

Revised Aim 1: ***Evaluate the utility of DNA sequences containing consecutive FdU nucleotides for intracellular delivery of Zn2+ to PSMA+ PCa cells*****. (Y1:Q1-4; Y2: Q1-3).**

Revised Task 1.A. Synthesize DNA hairpins with sequences containing FdU nucleotides and identify conditions useful for Zn2+ complex formation under physiological conditions. The stem region of the hairpin provides a model for the dimerization motif that will be used to create dimeric aptamer complexes.

a. Synthesize DNA hairpins with consecutive FdU nucleotides at the 3'-terminus, 5'-terminus, or alternating FdU-dA nucleotides for the stem region. **DNA hairpins will be chemically synthesized with FdU nucleotides site-specifically included either consecutively near the 5'- or 3'-terminus or alternating with dA nucleotides in the stem region**.

We have synthesized DNA hairpins having the properties required for these studies. A complete description of the synthesis, purification and spectral characterization of these DNA hairpins was

included in our recent publication that was reported in the previous annual progress report (Ghosh et al., 2011).

b. Use spectroscopic methods (CD, NMR, fluorescence) to demonstrate Zn2+ complex formation and evaluate the role of the FdU sequence in promoting Zn2+ complex formation.

We have completed spectroscopic evaluation and demonstrated Zn2+ complex formation occurs and that consecutive FdU nucleotides are required for Zn2+ complexes to form and have reported these novel findings (Ghosh et al., 2011).

c. Evaluate binding of netropsin to DNA hairpins with stems consisting of consecutive FdU and consecutive dA nucleotides and determine if netropsin binding facilitates Zn2+ complexation.

We recently completed 2D NMR and other studies demonstrating that netropsin binds to DNA hairpin sequences with stems consisting of consecutive FdU-dA base pairs (**Figure 1**). We have also demonstrated that netropsin binding to the 3'-FdU DNA hairpin sequence facilitates Zn2+ complexation (**Figure 2**). This work is highly novel in two fundamental aspects: i) these are the first studies demonstrating that netropsin binds DNA sequences with FdU-dA base pairs; ii) these are the first studies to demonstrate simultaneous binding in the minor and major grooves of DNA in order to deliver a therapeutic payload for cancer treatment.

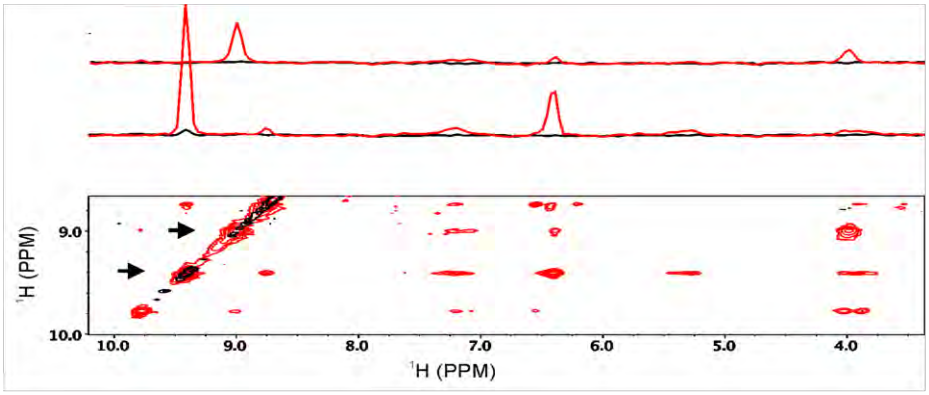


Figure 1. 2D NOESY spectrum demonstrating netropsin binds to the DNA hairpin 3'-FdU that contains 10 consecutive FdU nucleotides. This is the first demonstration that netropsin binds DNA sequences that include FdU-dA base pairs (from Ghosh et al., submitted).

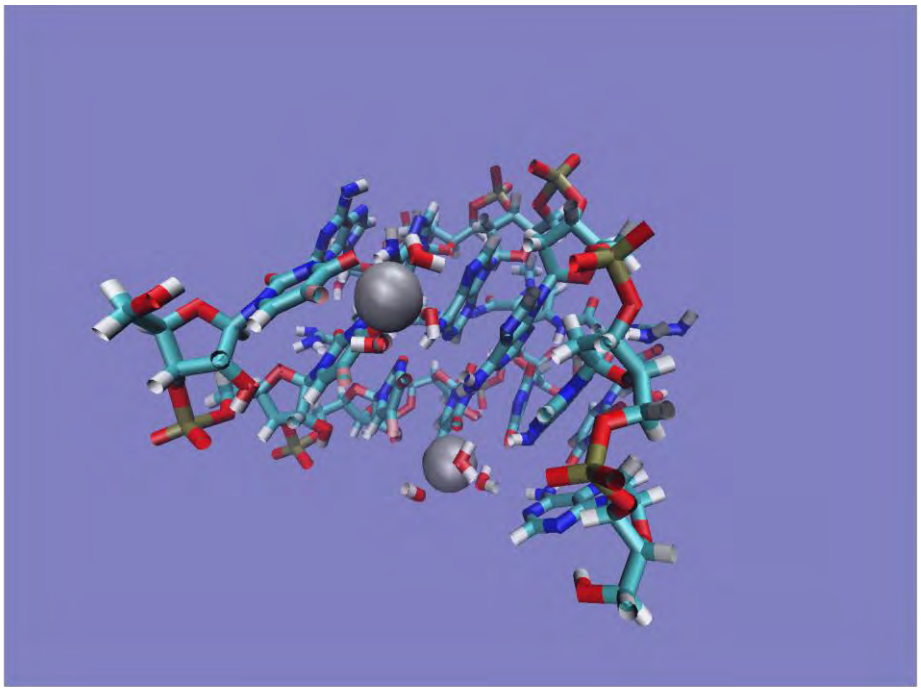


Figure 2. Molecular model of the Zn2+ complex with netropsin bound to FdU-substituted DNA. Zn2+ is shown as silver spheres. Two Zn2+ ions bind tightly to this complex.

This complex is highly novel from a structural biology viewpoint and it is also very important for this project because it demonstrates the feasibility of using this DNA structural motif to create the dimeric aptamer complexes that are the long-range goal of these studies. To further assess the therapeutic potential of this complex we have performed cytotoxicity assays with prostate cancer cells. Importantly, our studies have shown that the netropsin-stabilized Zn2+/DNA complexes are highly cytotoxic towards PC3 prostate cancer cells (**Figure 3**) indicating this motif is likely to be useful in creating the dimeric aptamer complexes that are the goal of these studies.

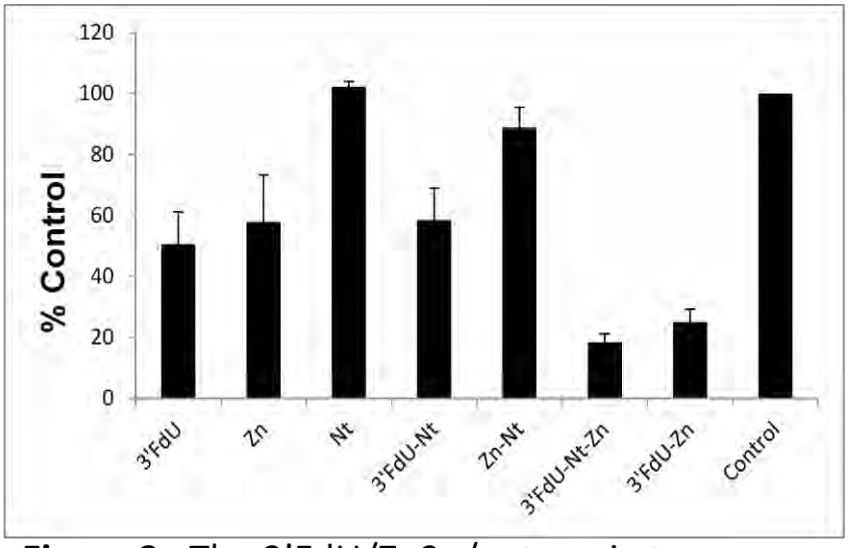


Figure 3. The 3'FdU/Zn2+/netropsin ternary complex displays enhanced cytotoxicity towards PC3 cells relative to the component moieties.

We expect to complete all studies with the Zn2+/netropsin/3'-FdU complex within the next month and to submit these studies for publication.

d. Evaluate FdU-dG base pair formation and determine if Zn2+ complexes with duplex DNA including FdU-dG base pairs are more stable under physiological conditions than duplex DNA including FdU-dA base pairs.

We have undertaken preliminary experiments with a DNA hairpin having a stem consisting of FdU-dG base pairs. The impetus for these studies is that the complexes involving A-FdU base pairs required pH slightly above physiological for long-term stability and thus were not ideal for *in vivo* delivery of therapeutics which is the long-term goal of these studies. G-FdU base pairs can form two hydrogen bonds even when FdU is deprotonated and we thought that this might allow such complexes to form and persist at physiological pH. Our preliminary studies indicate this is the case (**Figure 4**).

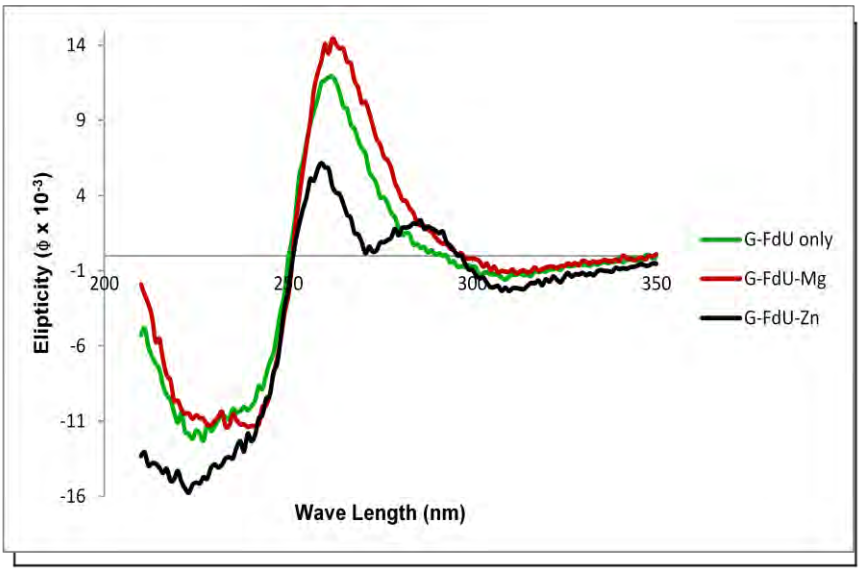


Figure 4. CD spectra indicating the DNA hairpin with G-FdU base pairs forms a complex with Zn2+ at physiological pH that differs in structure from that which forms either in the absence of divalent cations or in the presence of Mg2+.

Task 1.B. Evaluate the binding and internalization of these aptamer conjugates into prostate cancer cells and determine to what extent specific internalization is achieved into $PSMA^+$ cells. (Y1:Q3)

a. Cellular binding by fluorescence microscopy. The QST16 and QSA16 monomeric aptamers will be synthesized with fluorescent probes at their 5'-terminii. Binding to $PSMA^+$ (e.g. C4-2) and $PSMA^-$ (e.g. PC3) as monomers and as a dimer will be evaluated by fluorescence microscopy. (Y1: Q3)

We performed this study last year using the dT16 and dA16 aptamer complexes derived from the $SZTI_{PSMA01}$ aptamer. We did not include the quadruplex forming sequence element as we were in the process of developing the new Zn2+-binding motif. These studies showed strong binding to both PSMA+ and PSMA- cells (**Figure 5**). Thus, we did not have the required selectivity for PSMA+ cells and we re-initialized the SELEX procedure to identify a new DNA aptamer to PSMA.

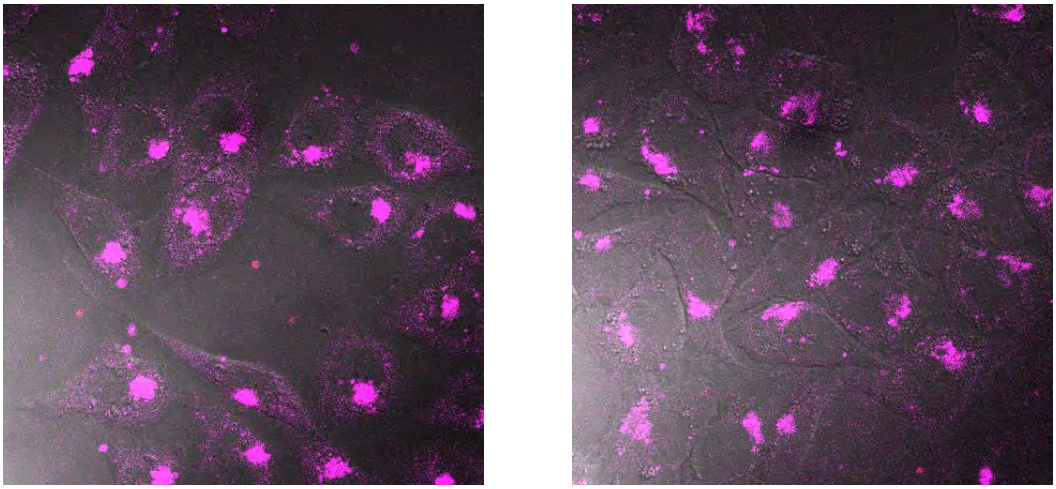
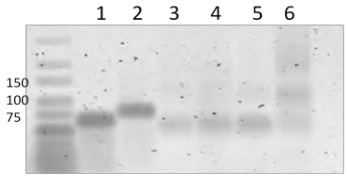
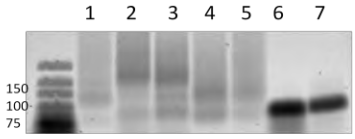


Figure 5. Confocal microscopy images showing cellular internalization of the dimeric aptamer complex composed of the $SZTI_{PSMA01}$ aptamer dA16 and dT16 sequence into PSMA-null cells (left) and PSMA-positive cells (right). Although strong uptake was observed in PSMA-positive cells (right), the uptake was not selective.

We re-initialized the systematic evolution of ligands through an exponential enrichment (SELEX) procedure to identify a new DNA aptamer to PSMA (**Figure 6**). One possible explanation for the lack of specificity was that the high affinity DNA sequence we identified previously was truncated relative to the length of the random insert used for the SELEX procedure. We have carefully analyzed the length of the insert during the selection procedure by closely following the mobility during each round of SELEX using electrophoretic gels. We also have synthesized a peptide that includes a His-tag sequence to remove sequences that bind to the His-tag on our PSMA construct used for the selection procedure. We are optimistic that these changes will permit us to identify a DNA aptamer for PSMA that has the desired selectivity for PSMA+ cells.



Lanes: 1. control 78mer
2. annealed 78mer
3. 4.5 single strand DNA in different round
6. double strand DNA (PCR) size ~120bp



Lanes: 1. double strand DNA (PCR) size ~120bp PCR-9th
2. ssDNA-9th
3. PCR-10th around (with GME bead)
4. ssDNA-10
5. ssDNA (prepared from upper band of PCR)
6. control 78mer
7. annealed 78mer

Figure 6. Representative gels from the SELEX procedure that we re-initialized.

We have completed the SELEX procedure and have identified the following DNA sequence as having high affinity for PSMA.

5'-dGGCCATAAGCGGTCACACAATCCCGTGATTCGCCTGCTA

We are completing other aspects of this project using this new aptamer sequence.

We used an RNA/DNA-folding algorithm program (Vienna RNA Fold; Grueber et al., 2008) to determine the likely secondary structure for this new aptamer (**Figure 7**). The identified sequence forms a stable hairpin with a minimum free energy of -9.50 kcal/mol.

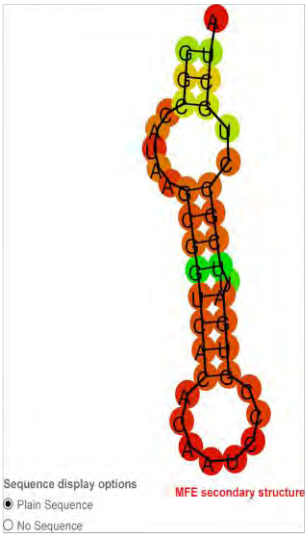


Figure 7. Secondary structure for the new aptamer sequence (displayed with U's, calculated with T's). The sequence identified forms a stable hairpin sequence.

b. Effects of Zn2+ complexation on cellular binding. The QST16 and QSA16 aptamer conjugates will form complexes with ZnPc and the effects of complex formation on cellular binding will be investigated using fluorescence microscopy. (Y1: Q4).

Our preliminary studies (**Figure 5**) indicated that dT-dA base pairing does not interfere with cellular binding or uptake. We still need to demonstrate that this is also the case for dimeric aptamer complexes with the new DNA aptamer and with the new Zn2+ binding motifs (e.g. FdU-dA, FdU-dG). At present, we are focusing on the binding of the new aptamer and making sure that this binding is selective for PSMA+ cells. Our preliminary microscopy studies (**Figure 8**) are consistent with the new aptamer binding selectively to PSMA+ cells.

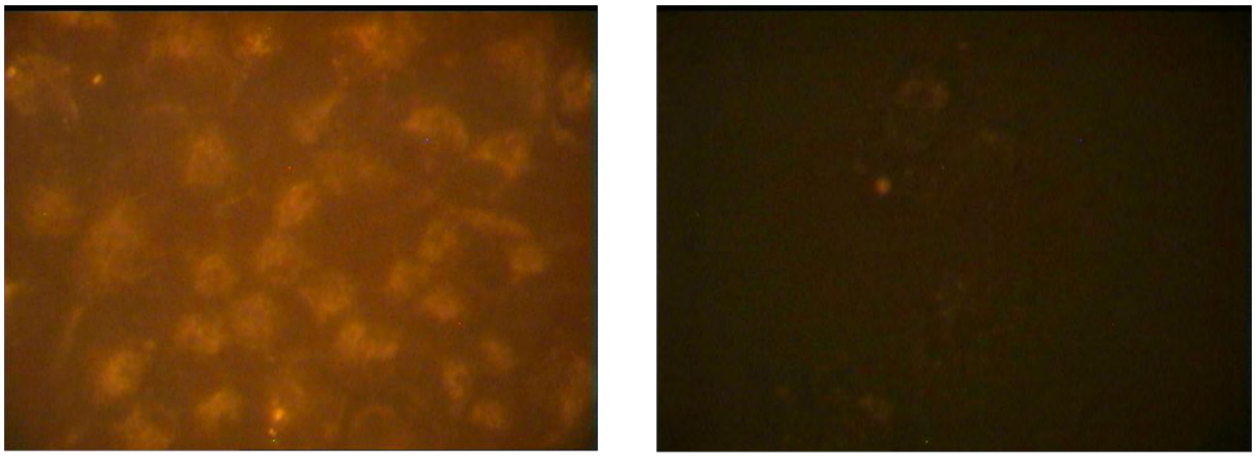


Figure 8. Selective binding of the new aptamer sequence to PSMA+ C4-2 cells (left) relative to PSMA- PC3 cells (right). The fluorescently labeled aptamer (1 μM) was incubated for 45 min at room temperature with the designated cell–type.

c. **Evaluate cellular internalization of aptamer complexes using confocal microscopy and flow cytometry**. The QST16 and QSA16 monomeric aptamer conjugates with fluorescent probes will be evaluated for cellular internalization into $PSMA^+$ and $PSMA^-$ cells using confocal microscopy and flow cytometry. The dimeric complex will be similarly analyzed. (Y1: Q4)

We have undertaken studies evaluating the internalization of the new aptamer sequence. Our studies are consistent with the new aptamer sequence undergoing selective internalization into PSMA+ cells (**Figure 9**).

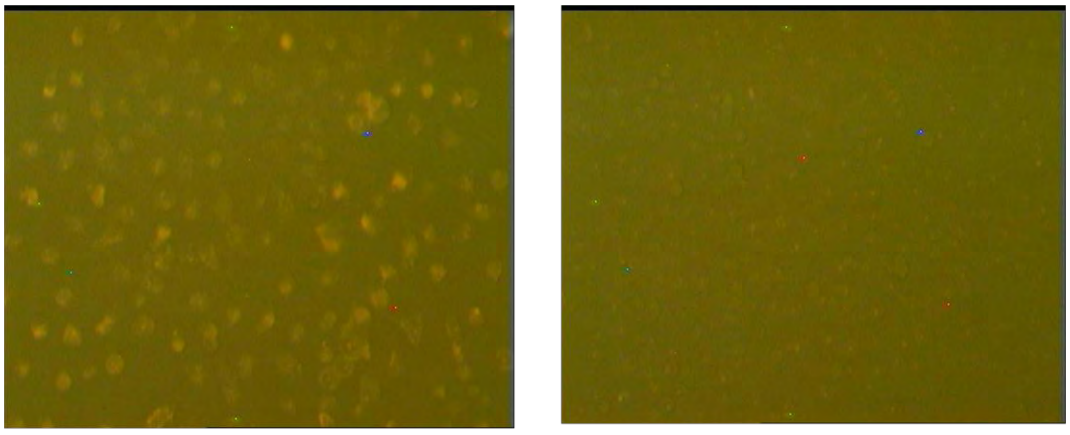
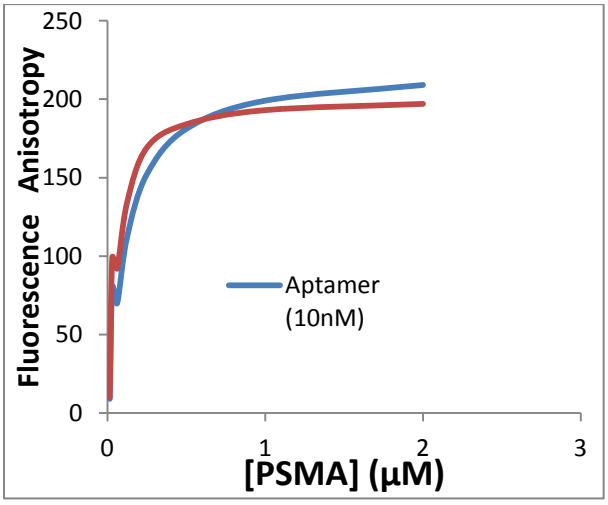


Figure 9. Selective internalization of the new aptamer sequence to PSMA+ PC3-pip cells (left) relative to PSMA- PC3-flu cells (right). The fluorescently labeled aptamer (0.3 nM) was incubated for 20 min at 37 °C with the designated cell–type.

Our microscopy studies indicate that this new aptamer sequence has the desired binding characteristics (binding selectively to PSMA+-cells at μM concentrations at room temperature and selective internalization into PSMA+ cells following incubation of nM concentrations at 37 °C. The binding and internalization of the aptamer for PSMA+ cells appears solid however the post-doctoral fellow that completed these studies (Tuya Sharkhuu) was dismissed from my laboratory and from Wake Forest School of Medicine and we are in the process of repeating these studies to assure their validity before moving forward with additional studies that require this aptamer sequence. We are in the process of interviewing candidates for a new post-doctoral fellow for this project and expect to make an offer soon to the individual that will continue this project.

One potential difficulty is that we have not yet been able to measure a binding constant for the new aptamer with recombinant PSMA. Our goal is to measure the binding constant and then to report our findings of the first DNA aptamer to PSMA. Attempts to measure the binding constant by fluorescence anisotropy, however, did not show PSMA-binding enhanced relative to a scrambled DNA sequence having the same nucleotide composition as the new aptamer. One potential reason for this is that the baculovirus-expressed PSMA we used for aptamer selection contained two His-tags sequences. We performed counter-selection against a peptide containing a His-tag sequence during SELEX, however it appears non-specific binding to the His-tag sequence is influencing our fluorescence anisotropy measurements. We plan to purchase a recombinant form of PSMA with zero or one His-tag sequences and perform these studies again.



d. Effects of ZnPc complex formation on cellular internalization. The effects of ZnPc complex formation on cellular internalization of monomeric and dimeric aptamer complexes will be evaluated using confocal microscopy and flow cytometry. (Y2: Q1).

Although we have not yet completed these studies, the new Zn2+-binding motifs we are developing as part of the revised SOW consist of FdU-dA and FdU-dG base pairs are unlikely to adversely affect cellular internalization as these motifs are far less obtrusive than the quadruplex motif for binding ZnPc that we originally proposed. We can complete these studies in only a month or so after we have clearly established the aptamer sequence is ready to commit the necessary resources for preparing the conjugates.

Aim 2: ***Evaluate the extent to which chelated Zn^{2+} (Zn^{2+}-pyrithione or ZnPc) enhances the cytotoxicity of fluoropyrimidine drugs (FPs) (5FU and FdUMP[10]) towards prostate cancer cells and determine if dimeric aptamer complexes containing both FPs and chelated Zn^{2+} display significantly greater cytotoxicity towards $PSMA^{+}$ PCa cells than complexes without FPs and/or without Zn^{2+}.*** **(Y1: Q1-4; Y2: Q1-4).**

Task 2.A. Evaluate the cytotoxicity of fluoropyrimidines in combination with Zn2+-chelates towards prostate cancer cells.

a. MTS assays. MTS assays will be used to assess the cytotoxicity of FPs (FdUMP[10] and 5FU) in combination with Zn^{2+}-chelates (Zn^{2+}-pyrithione and ZnPc). (Y1: Q1).

We have completed these studies and demonstrated that FdUMP[10] is highly cytotoxic towards prostate cancer cells and that the cytotoxicity of FdUMP[10] towards prostate cancer cells is clearly enhanced by Zn2+-pyrithione. We performed a more complete study evaluating FdUMP[10] from 10-5 to 10-9 M and Zn2+-pyrithione from 0 to 1 μM (**Figure 11**). For these studies, we used a cell-titer glo assay to evaluate effects of these drugs on cell viability. The cell-titer glo assay is similar to the MTS assay in that assesses cell viability and our laboratory has standardized on the use of this assay as it is fast and reliable. The results, plotted in **Figure 11**, demonstrate a positive interaction between zinc2+-pyrithione and FdUMP[10] at all concentrations tested.

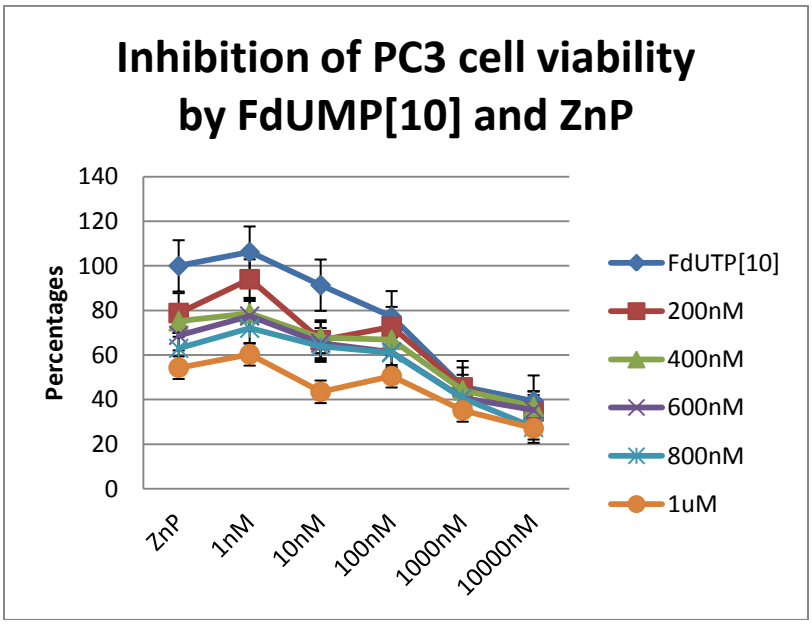


Figure 11. Results from a typical experiment demonstrating a positive interaction between FdUMP[10] and Zn2+-pyrithione (ZnPT). ZnPT markedly enhances the cytotoxicity of FdUMP[10] towards prostate cancer (PC3) cells.

b. Clonogenic assays. Clonogenic assays will be used to assess the effects of FPs and Zn^{2+}-chelates on the clonogenic survival of prostate cancer cells. (Y1:Q2).

We have used a modified clonogenic assay to evaluate the effects of Zn2+ on the cytotoxicity of our novel FdU-containing structures (**Figure 3**). Although clonogenic assays are useful they are time-consuming and reagent intensive thus our present studies focus on evaluating effects of Zn2+-pyrithione and FdUMP[10] on cell viability and apoptosis using cell-titer glo and caspase glo assays that are fast, reliable, and reasonably priced.

c. Evaluation of synergism. The MTS and clonogenic data will be analyzed using CalcuSyn to determine if the cytotoxicity of FPs and Zn^{2+}-chelates is synergistic, additive, or antagonistic. (Y1; Q3).

We have evaluated synergism using the cell-titer glo assay as input (**Figure 12**). FdUMP[10] and Zn2+-pyrithione have a positive interaction with the data overall supporting a synergistic interaction in PC3 cells.

CI For experimental values

Fd (nM)	ZnP (nM)	Fa	CI	
1	200	0.06	6.774	
1	400	0.21	1.692	
1	600	0.23	2.140	10^{-9} M
1	800	0.28	1.944	
1	1000	0.4	1.111	
10	200	0.33	0.368	
10	400	0.32	0.761	
10	600	0.35	0.927	10^{-8} M
10	800	0.36	1.154	
10	1000	0.57	0.413	
100	200	0.27	0.916	
100	400	0.33	0.922	
100	600	0.39	0.852	10^{-7} M
100	800	0.39	1.088	
100	1000	0.5	0.680	
1000	200	0.55	0.533	
1000	400	0.56	0.583	
1000	600	0.59	0.545	10^{-6} M
1000	800	0.59	0.617	
1000	1000	0.65	0.456	
10000	200	0.65	2.098	
10000	400	0.63	2.515	
10000	600	0.65	2.198	10^{-5} M
10000	800	0.73	1.145	
10000	1000	0.73	1.174	

Figure 12. Output from the program Calcusyn demonstrating that FdUMP[10] and Zn2+-pyrithione have positive interactions in PC3 cells with synergy over a broad range.

d. Evaluation of apoptotic cell death. A "live/dead" assay will be used to determine the extent of apoptotic cell death induced by FPs, Zn^{2+}-chelates, and the combination of FPs with Zn^{2+}-chelates. Western blots will be used to evaluate caspase and poly(ADP-ribose) polymerase (PARP) cleavage in drug-treated cells. (Y1: Q4).

We previously performed live/dead assays with FdUMP[10] in prostate cancer cells that demonstrated that FdUMP[10] induces apoptotic cell death. We have switched to the caspase-glo assay which is rapid and reliable. Our recent studies using the capsase-glo assay have not revealed induction of apoptosis by the Zn2+-pyrithione FdUMP[10] combination. There are several possible explanations for this: i) FdUMP[10]/Zn2+pyrithione induces a non-apoptotic form of cell death; ii) Apoptosis is rapid and caspase activity has peaked and attenuated before we have measured it; iii) Zn2+-pyrithione interferes with the assay reagent. We are presently sorting through these possibilities and expect to have definitive data in the next month.

Task 2.B. Evaluate the selective cytotoxicity of dimeric aptamer complexes containing FdU and/or ZnPc towards $PSMA^+$ prostate cancer cells.

a. Preparation of cytotoxic dimeric aptamer complexes. The dimeric aptamer complex will be prepared with FdU nucleotides by substituting FdU for dT in the dimerization motif. Complexes with ZnPc will be formed by incubation with ZnPc. Control complexes will contain neither FdU nor ZnPc. Specificity of the aptamer will be verified by using a scrambled aptamer sequence. Complex formation will be verified by CD and/or NMR spectroscopy. (Y2: Q1).

We have made good progress on the components for the dimeric aptamer complexes in that we have identified a new aptamer to PSMA and have established the utility of the FdU-dA and FdU-dG motifs for binding of Zn2+. We plan on preparing the dimeric aptamer complexes in the next 2 – 4 months after we have established the binding constant for the new aptamer with PSMA.

b. Specificity of dimeric aptamer complexes for PSMA$^+$ cells. PSMA-transfected and mock-transfected PC3 cells will be used to assess specificity of the dimeric aptamer complex for PSMA$^+$ cells. MTS and/or clonogenic assays will be used to assess cytotoxicity. (Y2: Q2-3).

We have perfomed studies evaluating the binding of the J591 mAb selectively to PSMA-transfected PC3 cells (PC3-pip) relative to mock-transfected cells (PC3-flu). Our data demonstrate that this cellular model is appropriate for these studies (Figure 14). We will use this cellular model to evaluate the dimeric aptamer complexes in the coming months.

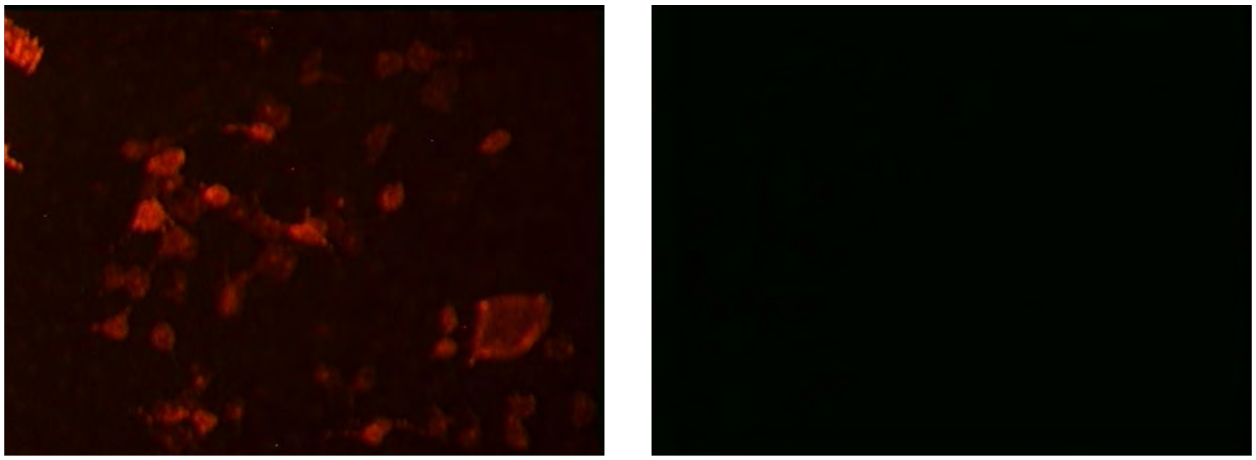


Figure 13. Selective binding of the J591 mAb to PSMA+ PC3-pip cells (left) relative to PSMA- PC3-flu cells (right). The mAb was incubated with the cells at 37 ^{o}C for 20 min.

c. Induction of apoptosis in PSMA$^+$ cells by dimeric aptamer complexes. Flow cytometry and Western blots will be used to investigate apoptotic cell death following exposure to dimeric aptamer complexes containing FdU and/or ZnPc. (Y2: Q3-4).

These studies have not yet been initiated although work on all the component parts is in progress such as the evaluation of apoptosis for FdUMP[10]/Zn2+-pyrithione.

Aim 3: ***Evaluate the safety and anti-tumor activity of chelated-Zn^{2+} in combination with advanced FPs as well as metallated DNA aptamer complexes that include both chelated Zn^{2+} and FdU.*** (Y2: Q1-4; Y3: Q1-4).

We have purchased animals to perform the safety study evaluating Zn2+-pyrithione *in vivo*. The animals are presently housed here in our AAALAC-accredited animal facility at WFSM and we plan to initiate treatment in the next few weeks. We will be following up with an efficacy study evaluating Zn2+-pyrithione in combination with FdUMP[10] and plan to evaluate the dimeric aptamer complex in the coming year.

KEY RESEARCH ACCOMPLISHMENTS:

- We have demonstrated that netropsin binds in the minor groove of DNA hairpins containing consecutive FdU nucleotides stabilizes Zn2+/DNA complexes. This is a unique DNA structural motif that promotes Zn2+ binding and delivery to prostate cancer cells. (Appendix 1).
- The novel netropsin/Zn2+/DNA complexes are highly cytotoxic to prostate cancer cells without a requirement for oligofectamine stabilization (Appendix #1). This new motif is likely to be useful for construction of dimeric aptamer complexes and delivery of Zn2+ to prostate cancer cells.
- We have demonstrated that DNA hairpins with stems consisting of dG-FdU base pairs bind Zn2+ under physiological conditions indicating that this structural motif may be preferred relative to FdU-dA base pairing for constructing dimeric aptamer complexes.
- We have identified a new DNA aptamer to PSMA and demonstrated selective binding to and internalization into PSMA+ prostate cancer cells. This is the first DNA aptamer to PSMA that we aware of and will be used for creating the dimeric aptamer complexes that are the goal of this project.
- We performed secondary structure calculations n the new DNA aptamer to PSMA that indicated that it forms a hairpin structure.
- We have demonstrated that FdUMP[10] is synergistic with Zn2+-pyrithione towards PC3 prostate cancer cells.
- A patent application (U.S. Patent Application No. 13/008,614 – Cytotoxic Nucleotides for Targeted Therapeutics) has been filed by the Office of Technology Asset Management at Wake Forest University.

REPORTABLE OUTCOMES:

Ghosh, S., Salsbury, F.R., Horita, D.A., Gmeiner, W.H. Zn2+ selectively stabilizes FdU-substituted DNA through a unique major groove binding motif. **Nucl Acids Res** 2011. PMID: 21296761.

U.S. Patent Application No. 13/008,614
Cytotoxic Nucleotides for Targeted Therapeutics
Inventor: William H. Gmeiner

CONCLUSION:

The dimeric aptamer complexes that deliver Zn2+ and FdU nucleotides specifically to prostate cancer cells in a PSMA-dependent manner have the potential to be highly efficacious and very well-tolerated by patients with advanced prostate cancer. We have demonstrated that the new Zn2+-binding DNA motif involving FdU-dA base pairs that we discovered is cytotoxic towards prostate cancer cells in a Zn2+-dependent manner and that netropsin stabilizes Zn2+ binding. Recent studies demonstrate that G-FdU base pairs also can be used to bind Zn2+. We expect one or more of these Zn2+-binding motifs will be useful in creating the dimaric aptamer complexes for this project. We have also identified a DNA aptamer sequence to PSMA. This is the first DNA aptamer to PSMA that we are aware of. We now have the requisite materials on-hand to construct the dimeric aptamer complexes. We have also established that the combination of FdUMP[10] and Zn2+-pyrithione is synergistic towards PC3 prostate cancer cells. We are now ready to initiate the in vivo aspect of this project. In summary, all aspects of this project are presently on track and this work is highly likely to result in a new reagent that useful for treatment of advanced prostate cancer.

REFERENCES:

Ghosh, S., Salsbury, F.R., Horita, D.A., Gmeiner, W.H. Zn2+ selectively stabilizes FdU-substituted DNA through a unique major groove binding motif. **Nucl Acids Res** 2011. PMID: 21296761.

Ghosh, S., Salsbury, F.R., Horita, D.A., Gmeiner, W.H. Cooperative Stabilization of Zn2+:DNA Complexes Through Netropsin Binding in the Minor Groove of FdU-Substituted DNA. In preparation. (Appendix #1).

Gruber AR, Lorenz R, Bernhart SH, Neuböck R, Hofacker IL. **The Vienna RNA Websuite.** Nucleic Acids Res. 2008

APPENDIX:

Manuscript In Preparation: February 2012

Cooperative Stabilization of Zn2+:DNA Complexes Through Netropsin Binding in the Minor Groove of FdU-Substituted DNA

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Abstract: The simultaneous binding of netropsin in the minor groove and Zn2+ in the major groove of a DNA hairpin that includes 10 consecutive FdU nucleotides at the 3'-terminus (3'-FdU) was demonstrated based upon NMR spectroscopy, circular dichroism, and computational modeling studies. The resulting Zn2+/netropsin:3'-FdU complex had very high thermal stability with aspects of the complex intact at 85 C, conditions that result in complete dissociation of Mg2+ complexes. CD and 19F NMR spectroscopy were consistent with Zn2+ binding in the major groove of the DNA duplex and utilizing F5 and O4 of consecutive FdU nucleotides as ligands with FdU nucleotides hemi-deprotonated in the complex. Netropsin is bound in the minor groove of the DNA duplex based upon 2D NOESY data demonstrating contacts between AH2 1H and netropsin pyrollidine (H3) 1H resonances. Netropsin binding induces a structural distortion that includes increase base stacking for consecutive adenine nucleotides. The Zn2+/netropsin:3'-FdU complex displayed increased cytotoxicity towards PC3 prostate cancer (PCa) cells relative to the constituent components or separate complexes (e.g. Zn2+:3'-FdU) indicating that this new structural motif may be therapeutically useful for PCa treatment.

Introduction

The coordination of metals by nucleic acids has important implications for biology, with metals such as Cd2+ and Ni2+ imparting toxicity and carcinogenesis while metals such as K+ and Mg2+ playing pivotal roles in nucleic acid structure and stability. Zn2+ plays a key role in cellular homeostasis in part through its coordination by Zn2+-finger peptidic motifs that are important regulators of protein-protein and protein-nucleic acid recognition. An intriguing observation is that Zn2+ levels vary dramatically during prostate cancer (PCa) carcinogenesis with normal prostate characteristically displaying the highest Zn2+ levels for any soft tissue in the human body while advanced PCa displays very low levels of Zn2+ with exogenous Zn2+ is cytotoxic to PCa cells and Zn2+-treatment sensitizes PCa cells to chemotherapy.

5-Fluoro-2'-deoxyuridine (FdU) is a non-native thymidine analog that has anti-cancer activity as a consequence of metabolism to the thymidylate synthase (TS) inhibitory metabolite FdUMP and the incorporation of the nucleotide triphosphate FdUTP into DNA that results in poisoning of DNA Topoisomerase 1. FdU is readily cleaved by glycosidase activities in vivo to 5FU and 5FU is widely used for treatment of diverse malignancies imparting a significant survival advantage for treatment of colorectal cancer. 5FU is inefficiently converted to FdUMP and FdUTP, however, and thus there is a need for alternative approaches to deliver DNA-directed fluoropyrimidine (FP) drugs. Our laboratory has pioneered the use of DNA polymers of FdUMP with

FdUMP[10] showing particularly strong cytotoxicity and a unique spectrum of activity in the NCI 60 cell line screen.

The chemosensitization of PCa cells by Zn2+ raises the possibility that Zn2+ chelates of FdU-oligodeoxynucleotides might be useful for treatment of advanced PCa. Zn2+ had been reported to form specific complexes with FdU-substituted DNA and coordination of Zn2+ by deprotonated FdU was proposed as the basis for specific complex formation. Zn2+-DNA complexes formed under similar conditions were however, found to be the result of inter-strand aggregation. We recently reported that Zn2+ formed specific complexes with FdU-substituted DNA hairpins provided the DNA sequence contained several consecutive FdU nucleotides. Biophysical characterization and computational chemistry approaches revealed Zn2+ bound FdU-substituted DNA in the major groove. Zn2+ was bound in a trigonal bipyramidal geometry with F5 from one FdU and O4 from an adjacent FdU serving as axial ligands for Zn2+ coordination. Zn2+ complexation required hemi-deprotonation of FdU nucleotides with F5 being activated for coordination by deprotonation. Hemi-deprotonation of the complex occurs near physiological pH consistent with the pKA for FdU (~7.6).

The occupancy of the major groove of DNA through Zn2+ complexation raises the possibility that Zn2+:DNA complexes could be further stabilized by binding of ligands in the minor groove. Minor groove binding may impart greater thermodynamic stability to the Zn2+:DNA complex and could potentially result in increased uptake into tumor cells through reduced charge density since minor groove binding ligands, such as netropsin

carry positive charges that partially offset the highly negative charge of the phosphodiester backbone of DNA. In this work, we demonstrate that netropsin and Zn2+ simultaneously occupy the minor and major groove the DNA hairpin 3'-FdU that includes 10 consecutive FdU nucleotides at the 3'-terminus. CD and NMR spectroscopy indicate the binding of Zn2+ occurs via a similar structural motif as occurs for the Zn2+:DNA complex while 2D NR spectroscopy verifies that netropsin binds in the minor groove formed from A-FdU base pairs similar to the binding motif established for A-T base pairs. The resulting Zn2+/netropsin:3'-FdU complex is highly cytotoxic towards PCa cells consistent with this unique structural motif being useful for PCa treatment.

Materials and Methods

Design and Synthesis of DNA Hairpins: The DNA sequence used for these studies (3'-FdU) was previously described and was designed to favor intramolecular hairpin formation and to be stable at physiological temperature. The hairpin DNA sequence consisted of a 10 base pair stem consisting of all A-FdU base pairs. The loop region included the GAA sequence that was previously shown to promote hairpin formation (11). The loop was closed by a single C.G base pair. Gel-electrophoresis studies demonstrated that intramolecular hairpins were selectively formed (supplementary data). The "3'-FdU" hairpin DNA sequence had the location of the ten consecutive A and FdU nucleotides with the 10 consecutive FdU nucleotides located at the 3'-terminus. The DNA sequences were prepared at the University of Calgary DNA Core laboratory and purified by gel-filtration chromatography.

Need to add netropsin info in the following sections:

Formation of Zn^{2+}:DNA Complexes: Intramolecular hairpins were formed using 1 mM DNA in dH_2O by heating to 80 °C for 5 min followed by rapid cooling. Native gel electrophoresis confirmed intramolecular DNA hairpin formation (Figure S). Zn^{2+} (or Mg^{2+}) complexes were formed by incubating 100 μM DNA with 0 to 5 mM $ZnCl_2$ (or $MgCl_2$) in M-DNA buffer (20 mM sodium cacodylate, pH 8.0, 10 mM NaCl) for 1 hour at room temperature. The formation of specific Zn^{2+} complexes with hairpin DNA sequences was evaluated using an EtBr exclusion fluorescence assay as previously described (5). This assay was performed by combining 20 μL of the above described M-DNA complex with 80 μL of EtBr solution (10 mM NaCl, 20 mM sodium cacodylate, pH 8.0, + 25 μM EtBr) followed by incubation for 30 min at room temperature in a 96 well clear bottom, black plate (Costar). The final divalent metal concentration ranged from 0 to 1 mM. EtBr fluorescence was detected using a Typhoon-9210 variable mode imager with Green laser (532nm) excitation and 610 nm emission filter (with 500 PMT set). Fluorescence intensity was quantified with the software ImageQuant5.2 and graphed vs Zn^{2+}/Mg^{2+} concentration.

Analytical Characterization of DNA:Zn^{2+} Complexes. DNA.Zn^{2+} complexes were analyzed by circular dichroism (CD) and NMR spectroscopy to characterize their structural features and any specific changes induced by Zn^{2+} or Mg^{2+}. 300 μL of 20 μM solution of DNA with either 0, 0.5mM, 1mM Zn^{2+} (or Mg^{2+}) were prepared in M-DNA buffer as described above and used to obtain the CD spectrum. An Aviv CD spectrometer was used to scan the region from 210 – 310 nm at a rate of 0.5 nm/sec. Temperature was controlled at 20 ^{o}C. For temperature-dependent CD studies,

temperature was increased from X to Y at a rate of Z C per minute. Spectra were acquired at 37, 70 and 85 C.

^{1}H, ^{19}F, ^{31}P, and ^{13}C NMR spectroscopy were used to investigate specific structural perturbations induced by Zn^{2+} to the 3'-FdU hairpin. For the NMR analysis, 500 µL of 0.5 mM 3'FdU in M-DNA buffer modified to contain 40 mM sodium cacodylate were used. NMR samples contained either no metal, 5 mM Zn^{2+} or 5 mM Mg^{2+}. 10% D_2O was included to maintain lock. The final pH of the samples was adjusted to either pH 7 or 8 (+/- 0.1 pH unit). ^{1}H NMR spectra were collected using a Bruker Avance DRX 600 MHz spectrometer with a TXI Cryoprobe at 10 °C using the WATERGATE sequence with a 3-9-19 refocusing pulse. Nulls were set at +/- 18.1 ppm. Spectra were acquired with a spectral width of 33.3 ppm, 8192 total data points, and 256 scans.

2D NMR experimental

^{19}F and ^{31}P NMR spectra were acquired using a Bruker 300 MHz NMR instrument with 85% H_3PO_4 (δ = 0 ppm) or 5mM aqueous solution of KF (δ=125.3ppm) as concentric internal standard capillaries (Wilmad).

Tm-analysis: Temperature-dependent melting of the 3'-FdU DNA hairpin complexed or not complexed with Zn^{2+}/Mg^{2+} and/or netropsin was determined using UV absorption spectroscopy at 260 nm. 400 µL of a 2.5 µM solution of the annealed hairpins in M-DNA buffer in the presence or absence of 0.5 mM Zn^{2+} (or Mg^{2+}) were analyzed using a

Beckman Coulter DU-800 UV-Vis spectrophotometer in the temperature region 20 – 85 ^{o}C. Temperature was increased at a rate of 0.5 ^{o}C/min. Melting temperatures were determined from the first derivative of the hyperchromicity profiles except for the 3'-FdU and 5'-FdU samples in the presence of Zn^{2+} for which an inflection point was not observed and the Tm calculated from the percent increase in hyperchromicity (see supplementary data).

Cytotoxicity of FdU Hairpins and Zn^{2+} Complexes. The cytotoxicity of the FdU hairpins and Zn^{2+} complexes was determined using a modified clonogenic assay with an MTT read-out. PC3 prostate cancer cells were seeded in 24 well plates at a cell density of 250 cells/well with 500 µL complete media (RPMI + 10%FBS + 1% Penicillin & Streptomycin). After 24 hours, media was removed and cells were washed twice with 500 µL PBS followed by addition of 500 µL serum-free RPMI. The 3'FdU-Zn^{2+} complex was formed by incubating 100 µM 3'FdU hairpin with 5mM Zn^{2+} in M-DNA buffer at room temperature for 1hour. Lipofectamine 2000 (Invitrogen) was diluted in 100µL Opti-MEM media (pH=8.0) to 7.5ng/µL and incubated at room temperature for 5 min. The 3'FdU-Zn^{2+} complex was then combined with diluted lipofectamine and incubated at room temperature for 20 min followed by the addition to each well. After 12 hours, media was replaced with fresh complete media and incubated at 37 ^{o}C for a total of 14 days with media changes every 2 days. Cell viability was then assessed using an MTS assay. All experiments were done in triplicate. Each set of data (net absorbance) were then expressed as a percentage, considering the no treatment (i.e. lipofectamine-only) group as 100%.

Computational Modeling. The initial models for the Zn^{2+} complexes were derived from coordinates for two consecutive dT-dA base pairs extracted from the NMR solution structure (pdb 1JVE)(12). Conversion to FdU was performed by deleting the thymidine methyl group and replacement with fluorine. Initially, eight complexes were geometry-optimized in the absence of Zn^{2+} and water molecules: 1) fully protonated: (both thymidine (or FdU) base nitrogens protonated), 2) hemi-deprotonated: one protonated thymidine (or FdU) base nitrogen and one unprotonated thymidine (or FdU) base nitrogen; 3) fully deprotonated: two unprotonated thymidine (or FdU) base nitrogens. There are two non-identical hemi-deprotonated complexes: i) 5'-dT- (or 5'-FdU-) deprotonated or ii) 3'-dT- (or 3'-FdU-) deprotonated). Geometry optimizations were performed with the addition of Zn^{2+} and water molecules to each complex. For all calculations, the NMR structure was used as the starting configuration and two Mg^{2+} counterions were added in the vicinity of the backbone phosphorus. Zn^{2+} was added (except in the control computation) between the fluorine atoms of the two FdU. Additional calculations confirmed the structures obtained were independent of initial Zn^{2+}-placement. All modifications were performed using Molden. All structures were geometry-optimized with Gaussian 03 using PM3 in Cartesian coordinates. Post-optimization, single-point calculations were performed with a minimal basis set (STO-3g*) to obtain energies and for an Natural Bond Orbital analysis(13) from which the Wieburg bond orders were extracted. Stabilization energies were calculated by subtraction from the energy of the full complex the energy of dA-dT (or FdU) in the

appropriate protonation state (doubly, singly, or unprotonated), and the energy of Zn^{2+} with four water molecules.

RESULTS

Stabilization of Zn2+:DNA Complexes through Netropsin Binding: We have previously established that DNA sequences consisting, in part, of consecutive dA-FdU base pairs, such as the DNA hairpin sequence 3'-FdU (Figure 1) form highly stable complexes with Zn2+. Zn2+ complex formation occurs via major groove binding to the DNA duplex and requires hemi-deprotonation of the FdU imino hydrogens. As netropsin is known to bind in the minor groove of duplex DNA selectively at runs of consecutive dA-dT base pairs, we sought to determine if netropsin would also bind to consecutive dA-FdU base pairs and, if so, to determine to what extent netropsin binding (likely in the minor groove) would cooperatively stabilize Zn2+-binding in the major groove of the 3'-FdU sequence. The minor groove atomic configuration for FdU-substituted DNA sequences, such as the 3'-FdU DNA hairpin, is identical to that for DNA sequences consisting of dA-dT base pairs making it likely that netropsin would bind in a similar manner. Further, netropsin binding would be expected to reduce base pair dynamics for DNA complexes. This reduced flexibility could result in cooperative enhancement of Zn2+-binding in the major groove if the preferred geometries for DNA in the netropsin complex and the Zn2+ complex were similar.

The propensity for netropsin binding to alter the formation and stability of Zn2+ complexes with the 3'-FdU hairpin were studied using EtBr exclusion assays (Figure 1) and UV (Figure S1) and CD (Figure 2) thermal melting studies. We have previously shown that EtBr intercalation into the 3'-FdU sequence is inhibited by Zn2+ complexation, but is not blocked by Mg2+ binding. Netropsin binding alone inhibited EtBr intercalation consistent with netropsin binding the 3'-FdU hairpin sequence and stabilizing the duplex in a manner that prevents EtBr intercalation. Consistent with our previous results, Zn2+-binding alone inhibited EtBr intercalation. Interestingly, the combination of Zn2+ and netropsin was more effective at inhibiting EtBr intercalation than was either agent alone, consistent with cooperativity in the binding of these moieties to distinct sites on the DNA duplex (Figure 1).

The thermal stability of the 3'-FdU complex with Zn2+ and netropsin was further investigated using UV hyperchromicity (Figure S1) and temperature-dependent CD spectroscopy (Figure 2). We had previously demonstrated that Zn2+ complexation resulted in a substantial increase in thermal stability for the 3'-FdU DNA hairpin while Mg2+ binding had a more moderate stabilizing effect. UV hyperchromicity measurements revealed that the sigmoidal shaped curve typically observed in such experiments did not occur for the Zn2+/netropsin:3'-FdU complex although such sigmoidal curves were detected for the Mg2+/netropsin:3'-FdU complex and the netropsin:FdU complex (Figure S1). The overall increase in absorbance (260 nm) for both the Mg2+/netropsin:3'-FdU complex and the netropsin:FdU complex was the 20-25% that is typical for melting duplex DNA and both complexes had similar Tm values.

In contrast, the Zn2+/netropsin:3'-FdU complex displayed a maximum increased absorbance of about 15% indicating strand dissociation was not complete, even at temperatures above 80 C. The results indicate that Zn2+ and netropsin may cooperatively stabilize 3'-FdU and that the Zn2+/netropsin:3'-FdU complex likely undergoes step-wise dissociation with the complex remaining incompletely dissociated even at elevated temperatures.

CD Spectral Analysis: The structure and stability of the Zn2+/netropsin:3'-FdU complex was further evaluated by CD spectral analysis including temperature-dependent studies, to determine to what extent Zn2+ and netropsin binding, both singly and in combination, alters DNA structure and to gain further insight into the thermal stability of the complex (Figure 3). Our previous studies demonstrated that Zn2+, but not Mg2+, induced a concentration-dependent blue-shift of approximately 20 nm for the maximal positive ellipticity (280 -> 260 nm) for the 3'-FdU sequence. The effects of netropsin binding to 3'-FdU are similar to that observed previously for binding to A-T rich DNA duplex sequence and involve a slight red-shift for the maximal positive ellipticity (280 -> 285 nm) a sharp new maximum at 260 nm, and a broad new maxima at approximately 320 nm. The red-shift for the maximal ellipticity and the new maxima at 260 nm likely involve slight structural alterations for the A-FdU base pairs to accommodate netropsin binding in the minor groove while the new 320 maxima reflect the binding orientation of the N-methyl pyrrolidine moieties of netropsin bound in the minor groove of 3'-FdU.

The effects of Zn2+ and Mg2+ binding to the CD spectra of the 3'-FdU:netropsin complex revealed highly specific alterations to the structure and stability of the complex were induced by Zn2+, but not Mg2+. Mg2+ binding to the 3'-FdU:netropsin complex resulted in moderate attenuation of the maxima at 260, 280, and 320 nm, however Mg2+ binding resulted only in negligible shifts in the maxima location. In contrast, Zn2+ binding resulted in a substantial enhancement for the maxima at 260 nm (~4-fold increase relative to netropsin:3'-FdU) and a marked reduction in the maxima at 320 nm. The enhancement for the maxima at 260 nm likely reflects Zn2+-induced structural perturbations to the FdU nucleobases that are re-enforced by netropsin binding. The thermal stability of 3'-FdU complexes with netropsin and/or Zn2+ (or Mg2+) were further investigated by temperature-dependent CD spectroscopy. The maxima at 260, 280, and 320 nm were reduced by approximately 50% upon increasing the temperature from 37 to 70 C. Further temperature increase to 85 C resulted in near complete loss for these maxima. The temperature sensitivity for CD spectra of the 3'-FdU-Mg2+ complex were nearly indistinguishable from netropsin:FdU indicating Mg2+ had little effect on the thermal stability of the complex. In contrast, the 260 nm maxima for the Zn2+/netropsin:3'-FdU complex was attenuated by only ~10% upon increasing the temperature from 37 to 70 C and the intensity for this maxima was reduced only about 30% upon increasing the temperature to 85 C. The results are consistent with Zn2+ and netropsin binding cooperatively to 3'-FdU and resulting in formation of a highly stabilized complexed.

NMR Spectroscopy: The interactions of Zn2+ and netropsin with 3'-FdU in the context of the Zn2+/netropsin:3'-FdU complex were investigated using NMR spectroscopy. Our previous studies with the Zn2+:3'-FdU complex revealed significant downfield shifts for approximately one-half of the 19F resonances relative to 3'-FdU in the absence of Zn2+. Subsequent computational and modeling studies indicated that F served as one ligand for Zn2+ coordination in the Zn2+:3'-FdU complex and that F was activated through deprotonation of the FdU imino 1H which occurred for about one-half of FdU nucleotides under the experimental conditions used (hemi-deprotonation of FdU nucleotides in the Zn2+:3'-FdU complex). 19F NMR spectra for the Zn2+/netropsin:3'-FdU revealed similar downfield shifts occurred for this complex consistent with Zn2+ coordination involving F in this complex, as well. In contrast, no significant changes were noted in the 19F NMR spectrum for the Mg2+/netropsin:3'-FdU complex and netropsin alone did not induce such changes to the 19F spectrum. 31P NMR spectra indicated no significant change upon Zn2+ and/or netropsin binding to 3'-FdU although changes were detected for Mg2+/netropsin consistent with Mg2+, but not Zn2+, interacting with the phosphodiester backbone of 3'-FdU in a non-specific manner.

The interactions of Zn2+ and netropsin with 3'-FdU were further analyzed by 1D and 2D 1H NMR spectroscopy. Imino 1H resonances for FdU nucleotides were readily detected at characteristic frequencies for the Zn2+/netropsin:3'-FdU complex consistent with our previous studies indicating hemi-deprotonation, rather than full deprotonation, was required for complex formation (Figure). 2D NOESY spectra were obtained to gain insight into the binding of netropsin to 3'-FdU (Figure). The binding of netropsin or other

putative minor groove bindings ligands at A-FdU (rather than A-T) base pairs had not been described previously, however our 2D NOESY studies demonstrate netropsin interacts with 3'-FdU in the minor groove. NOESY crosspeaks were observed between resonances with chemical shifts consistent with an AH2 resonance asignment and those consistent with the pyrrolidine H resonances of netropsin. NOESY crosspeaks from FdU imino 1H to putative AH2 resonances were not detected, however, preventing unambiguous assignment of the AH2 resonances. The lack of imino 1H – AH2 resonances likely results from reduced intensity for FdU imino 1H as a consequence of hemi-deprotonation of FdU in the Zn2+/netropsin:3'-FdU complex. Interestingly two AH2-AH2 crosspeaks were present for the Zn2+/netropsin:3'-FdU complex that were not observed for the Zn2+:3'-FdU complex (Figure). The presence of AH2-AH2 crosspeaks is consistent with increased p-p stacking for A residues in the context of Zn2+/netropsin complex. Such a conformational change is also consistent with the CD spectral data that indicated significant structural perturbations occurred for the DNA nucleobases in the context of the Zn2+/netropsin:3'-FdU complex relative to 3'-FdU alone.

Cytotoxicity of Zn2+/netropsin:3'-FdU to PC3 Cells. Our previous studies demonstrated that 3'-FdU is cytotoxic to PC3 prostate cancer cells and that Zn2+ complexation increased the cytotoxic effects. In these studies, the Zn2+:3'-FdU complex was delivered to cells using oligofectamine both to enhance cellular permeability and because the complex was labile under conditions used in the tissue culture experiments. In light of the increased thermal stability for Zn2+/netropsin:3'-FdU

relative to the Zn2+:3'-FdU complex we sought to determine if this complex might display increased cytotoxicity towards PCa cells. Cytotoxicity was determined using a modified clonogenic assay with an MTS assay readout. Under the conditions used for these experiments the 3'-FdU hairpin and the Zn2+:3'-FdU complex displayed similar cytotoxicity consistent with dissociation of Zn2+ which had moderate cytotoxicity as a single agent (Figure). The netropsin:3'-FdU complex displayed slightly lower cytotoxicity than 3'-FdU, and netropsin as a single agent actually increased viability relative to control cells. These results indicate netropsin is growth stimulatory towards PCa cells, a surprising finding in light of the antitumor/antibiotic activity described previously for netropsin. The Zn2+/netropsin-3'-FdU complex displayed enhanced cytotoxic effects relative to the individual components (Zn2+, netropsin, 3'-FdU) and relative to Zn2+:3'-FdU complex. In light of the proliferative effects of netropsin, these findings demonstrate that netropsin is likely enhancing the cytotoxicity of the Zn2+/netropsin:3'-FdU complex by stabilizing the complex and possibly through enhancing cellular uptake.

Computational Chemistry Studies of Zn2+/netropsin:3'-FdU.

(Fred – your results will go here).

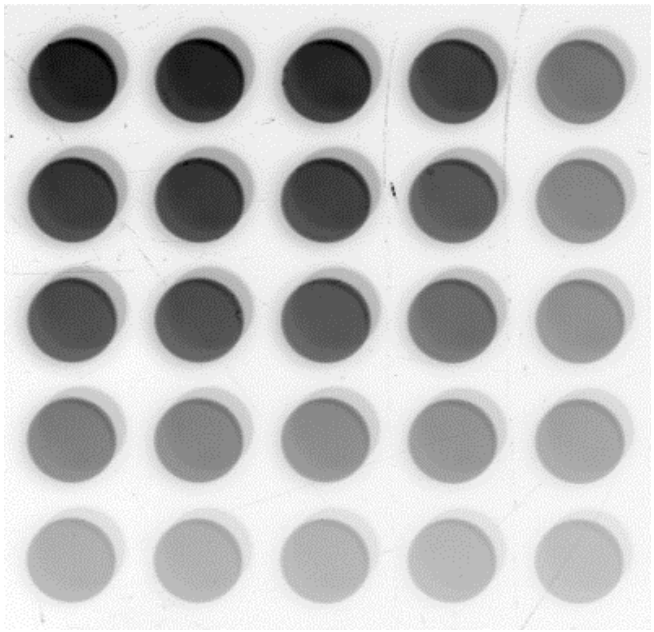
Discussion:

The present work demonstrates that Zn2+ and netropsin can simultaneously bind DNA hairpin sequences containing consecutive FdU nucleotides with Zn2+ occupying the major groove and netropsin binding the minor groove of the DNA double helix. The evidence in support of the unique structural configuration for this complex includes CD spectral data which are consistent with perturbations to the DNA nucleobases occurring in a Zn2+- and netropsin-dependent manner. The NMR spectral data also were consistent with the major groove binding motif for Zn2+ previously established based on NMR and computational chemistry data from our laboratory and with netropsin binding in the minor groove based upon NOE data from pyrrolidine H 1H of netropsin and AH2 1H resonances in the minor groove of the 3'-FdU hairpin.

The simultaneous binding of netropsin and Zn2+ to 3'-FdU resulted in significant stabilization of the complex. The thermal stability of the Zn2+/netropsin:DNA complex could not be completely assessed by UV hyperchromicity measurements because complete strand dissociation was not achieved, even for the highest temperatures used for these studies (~85 C). Temperature-dependent CD data demonstrated that the complex remained largely intact at 70 C and remained at least partially present at 85 C. This high thermal stability was found to correlate with improved cytotoxicity consistent with the complex persisting under physiological conditions for times sufficient for cellular uptake. The improved stability for the Zn2+/netropsin:3'-FdU may be useful for in vivo studies and result in increased circulation times for the intact complex relative to linear

homopolymers of FdUMP, such as FdUMP[10] which has shown promise in pre-clinical studies for cancer treatment.

The structural basis for the high thermal stability of the Zn2+/netropsin:3'-FdU complex appears to arise from cooperative binding. Both netropsin-only and Zn2+-only binding to 3'-FdU induce a new maxima at 260 nm in CD spectra, likely as a result of perturbations in the structure of the FdU nucleobases. This maxima is significantly enhanced upon simultaneous binding of Zn2+ and netropsin consistent with cooperativity among the two types of binding. The NMR spectral data indicate that Zn2+ complexation occurs in a manner similar to that observed for the Zn2+:3'-FdU complex (computational studies also support?) while NMR data indicate netropsin binds in the minor groove making NOE contact with AH2 resonances from A-FdU base pairs. NOESY spectra also demonstrate base stacking, manifested by AH2-AH2 NOEs occur upon netropsin and netropsin/Zn2+ binding to 3'-FdU. The binding of netropsin is similar to that which occurs for duplex DNA with consecutive A-T base pairs however the base stacking features detected by NMR and consistent with the CD spectral data indicate netropsin binding induces structural changes in 3'-FdU that re distinct from that previously described for A-T-rich sequences.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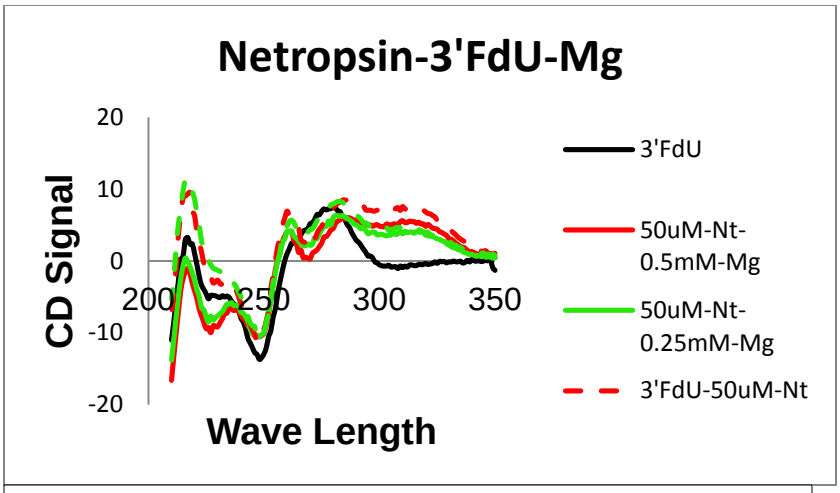
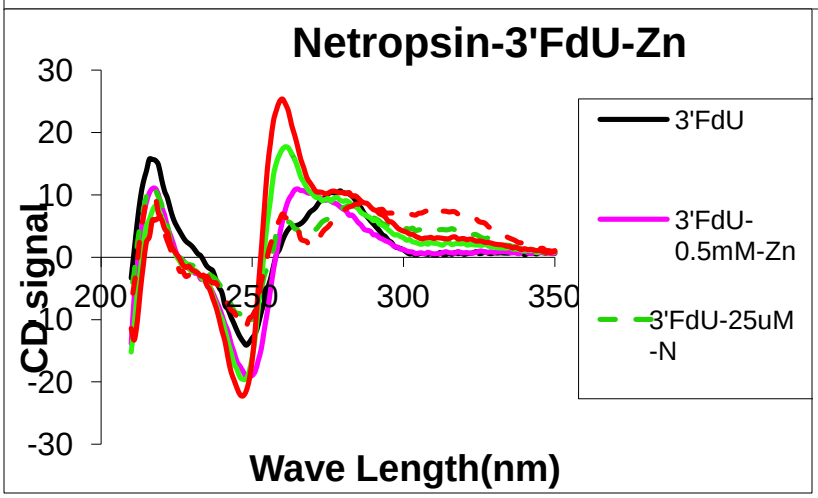
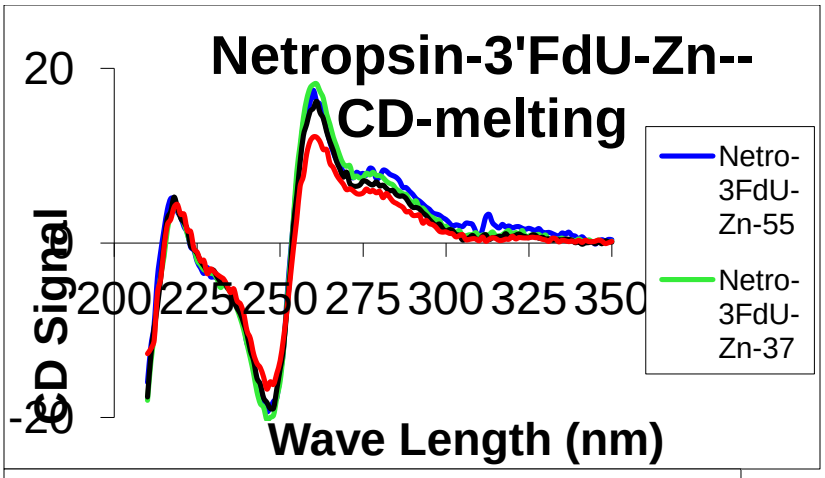
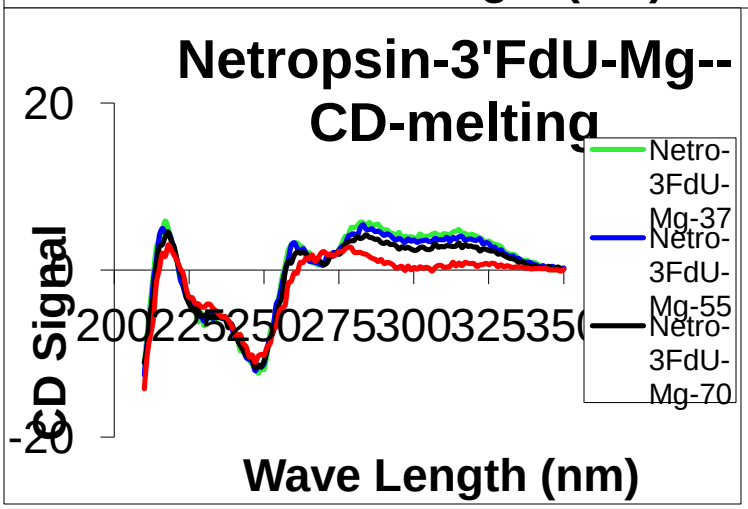
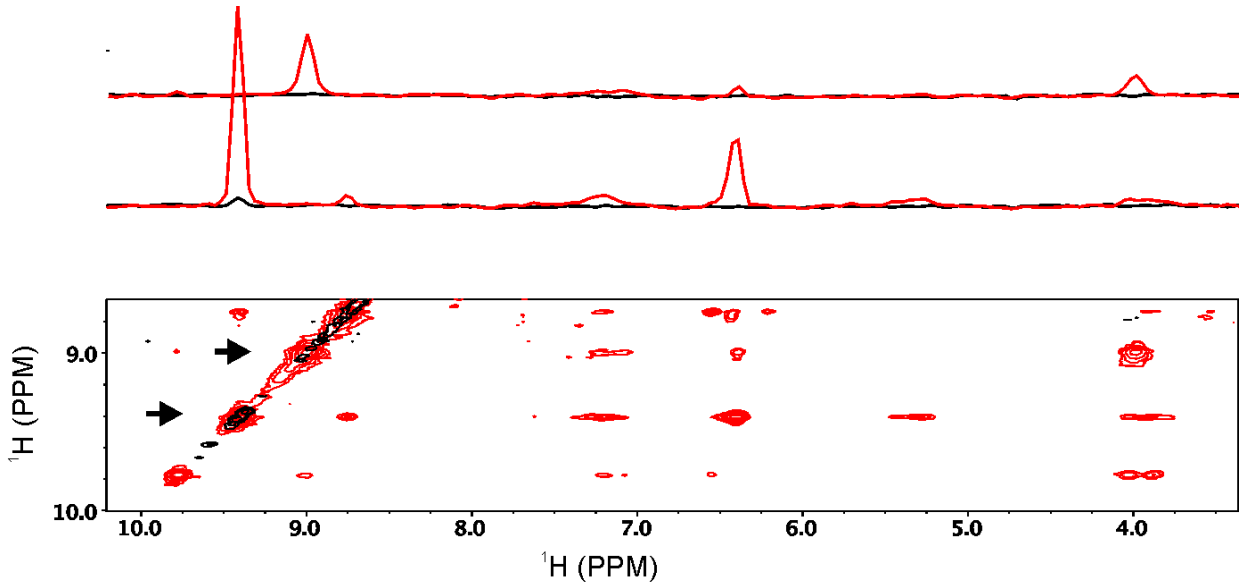
10
25
50


Netropsin-3'FdU-Mg
CD Signal
20
10
0
-10
-20
200
250
300
350
Wave Length
3'FdU
50uM-Nt-0.5mM-Mg
50uM-Nt-0.25mM-Mg
3'FdU-50uM-Nt


Netropsin-3'FdU-Zn
CD signal
30
20
10
0
-10
-20
-30
200
250
300
350
Wave Length(nm)
3'FdU
3'FdU-0.5mM-Zn
3'FdU-25uM-N


Netropsin-3'FdU-Zn--CD-melting
20
0
-20
200 225 250 275 300 325 350
CD Signal
Wave Length (nm)
Netro-3FdU-Zn-55
Netro-3FdU-Zn-37


Netropsin-3'FdU-Mg--CD-melting
20
0
-20
CD Signal
Wave Length (nm)
Netro-3FdU-Mg-37
Netro-3FdU-Mg-55
Netro-3FdU-Mg-70


9.0
10.0
^{1}H (PPM)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
^{1}H (PPM)